DIAGNOSTIC MARKERS FOR THERAPEUTICAL TREATMENT

FIELD OF THE INVENTION

This invention relates to the field of diagnosis and in particular to biological markers associated with disease states, and associated with therapeutical treatment.

PRIOR ART

The following is a list of prior art which is considered to be pertinent for describing the state of the art in the field of the invention. Acknowledgement of these references herein will be made by indicating the number from their list below within brackets.

(1) Olah M.E. and Stiles G.L. The role of receptor structure in determining adenosine receptor activity, *Pharmacol. There.*, **85**:55-75 (2000);

(2) Poulsen S.A. and Quinn R.J.,Adenosine receptors: new opportunities for future drugs. *Bioorg. Med. Chem.*, **6**:619-641 (1998);

(3) Fang X. *et al.* Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A., *Proc. Natl. Acad. Sci.* USA, **97**:11960-11965 (2000);

(4) Fishman, P., *et al.*, Involvement of Wnt Signaling Pathway in IB-MECA Mediated Suppression of Melanoma Cells, *Oncogene* **21**:4060-4064 (2002);

(5) Ferkey, D.M., and Kimelman, D. *GSK-3:* New Thoughts on an Old Enzyme, *Dev. Biol.*, **225**:471-479 (2000);

(6) Bonvini, P., *et al.* Nuclear beta-catenin displays GSK-3beta- and APC-independent proteasome sensitivity in melanoma cells, *Biochim. Biophys. Acta.*, **1495**:308-318 (2000);

(7) Olah, M.E. and Stiles, GL, The role of receptor structure in determining adenosine receptor activity, *Pharmacol. Ther.*, **85**:55-75 (2000).

BACKGROUND OF THE INVENTION

A_3 adenosine receptors belong to the family of the Gi-protein associated cell surface receptors. Receptor activation leads to its internalization and the subsequent inhibition of adenylyl cyclase activity, cAMP formation and protein kinase A (PKA) expression, resulting in the initiation of various signaling pathways [1,2]. PKA contains a catalytic subunit PKAc which dissociates from the parent molecule upon activation with cAMP. Recent studies have demonstrated that PKAc phosphorylates and inactivates a GSK-3β [3].

Recently, it has been shown that 1-deoxy-1-[6[[(3-iodophenyl)methyl] amino]-9H-purine-9-yl]-N-methyl-β-D-ribofura-nuronaminde (IB-MECA), a stable agonist to A3AR, alters the expression of GSK-3β and β-catenin, key components of the Wnt signaling pathway. Consequently it let to the inhibition of the expression of the cell cycle progression genes, c-myc and cyclin D1 [4].

SUMMARY OF THE INVENTION

The present invention is based on the finding that agonists of the A_3 adenosine receptor (A3AR) alter several characteristics of cellular markers, including their expression level, phosphorylation, and their cellular localization . Thus it is possible to assess, or monitor the success of a therapeutical treatment by A3AR modulators by monitoring the change of the expression, phosphorylation, or localization of a number of biological markers.

Thus present invention concerns a method for monitoring the effectiveness of a treatment against a disease state, by monitoring, continuously or once , the level of at least one parameter of a biological marker as compared to a control level, the control level being the level of the parameter without treatment, which is determined either as the level of the parameter in the individual prior to treatment or the level of the parameter in untreated control having the disease state. The method comprising :

(a) obtaining a sample of cells, or tissue, associated with the disease from a subject having a disease state and being administered with a drug against the disease;

(b) detecting the level of at least one physiological parameter of at least one biological marker in said cells, the marker being an A3AR,or an element associated with the A3AR signal transduction pathway which is downstream to A3AR; and

(c) comparing the level of said at least one parameter of at least one marker to the level thereof in untreated cells being cells from the same subject before administration of said drug ,or being a standard reference for said marker which is indicative of an un-treated disease state;

wherein a difference in level of the physiological parameter the treated and untreated cells between indicative of the effectiveness of said treatment against the disease state.

Typically the treatment is the administration of an A3AR modulator being an A3AR agonist or antagonist, most preferably an A3AR agonist such as IB-MECA.

The term "***biological marker***" or in short "***marker***" according to the invention should be construed in its broad sense to refer to any endogenous, preferably, cell associated, substance, including, without being limited thereto, an amino acid compound (e.g. protein, polypeptide or peptide) nucleic acid compound (e.g. mRNA), cell metabolite, which are either present at the surface of the diseased cells (e.g. cell surface receptor, cell surface glycoproteins, etc.), shedded or secreted

from the cell or which is present within the cell (e.g. a kinase). In particular, the biological markers are markers associated with the signal transduction pathway of A3Ar i.e.. elements that are known or that are experimentally found to be associated with the signal transudation mediated by A3AR . Examples are elements of the Wtn signal transudation pathway including: and include all components of PKA, PKB, GSK-3β, , β-catenin, cyclin D1 and c-myc. And elements of the NF-κB signal transudation pathway such as , PI3K,, IKK,,IKB,NF-κB, cyclin D1 and c-myc

The term ***"the level of a physiological parameter"*** refers to one of the following :

(1) The level of the biological marker's expression as determined by the amount of the biological marker's protein or protein fragment, or as determined by the amount of the biological marker's mRNA . This parameter is relevant to all markers namely: A3AR, PKA, PKB, GSK-3β, NF-$_{\kappa}$B, cyclin D1, β-catenin).

(2) The phosphorylation level of the biological marker, This parameter is relevant to PKB, PKA and especially to GSK-3β and beta-catenin).

(3) The cellular localization of the biological marker, for example localization in cell membrane vs. cytosolic (for A3AR), cytosol vs. nucleous (β-catenin, NF-kappaB)..

Detecting the level of expression of the biological marker is carried out by any technique known in the art to detect the presence of a protein or a fragment of a protein in cells either at the cytosol or the membrane as well as by techniques for the detection of mRNA using any technique known in the art to detect the presence of a protein, or a fragment of a protein in cells either at the cytosol, at the membrane, or in any intracellular component of the cells, as well as in techniques for the detection of mRNA level in any component of the cells.

Methods for detecting the level of the protein may include: extracting the protein contents of the cells, or extracting fragments of protein from the membranes of the cells, or from the cytosol, for example, by using sate of the art

lysis, digestive, separation, fractionation and purification techniques, and separating the proteinaceous contents of the cells (either the crude contents or the purified contents) on a western Blot, and then detecting the presence of the protein , or protein fragment by various identification techniques known in the art. For example the contents separated on a gel may be identified by using suitable molecular weight markers together with a protein identification technique, or using suitable detecting moieties (such as labeled antibodies, labeled lectines, labeled binding agents (agonists, antagonists, substrates, co-factors, ATP, etc). The detection may also be by in situ, i.e. in the full tissue sample, by binding of specific recognition agents, to the biological markers when present in intact cells or in-tissue, (relevant in connection with the present invention especially to determination of the level of A3AR) .Such specific recognition agents may be labeled A3AR agonists, such as labeled IB-MECA; labeled A3AR antagonists such as labeled MRS1523, antibodies against A3AR etc. The presence of the labeled recognition moieties may be detected using techniques suited for the nature of the label.. Where the recognition agents are fluorescent-labeled , the detection may be carried out by using a confocal microscope and directly viewing the level of the of the label bound (to the membranes). Where the recognition agents are labeled, for example, radio-labeled, the level may be determined by the determination of the radio-label level in the cells.

The determination of expression level may also be determination of mRNA level, for example, the detection may be by any methods used in the art for the detection of RNA in a cell-containing sample such as by using *in situ* hybridization with a detectable probe, for example, with a complementary sequence containing a detectable moiety (fluorescent, radioactive, chromatophoric moiety, etc). In such a case of *in situ* hybridization there is no need to extract the RNA from the cells and all that is needed is treatment to render the cells porous. However various amplification methods, which, are sensitive enough to detect to minute amounts of RNA are preferable. Such methods include, PCR, RT-PCR, *in situ* PCR, *in situ* RT-PCR (all the above referring also *to "nested"* PCR, and nested RT-PCR), LCR

(ligase chain reaction) and 3SR (self sustained sequence replication). In accordance with a preferred embodiment RT-PCR and nested RT-PCR are used. The amplification products are identified by methods used in the art such as by separation on a gel and detection using a suitable labeled probe.

The sample may be membranes of tissue samples for example obtained by biopsy , in tact cells separated from the tissue sample, or intact cells present in the circulation such as in the blood or any other body fluid, cells,or tissue samples obtained from the subject including paraffin embedded tissue samples, proteins extracted obtained from the cytosol, cell membrane, nucleus or any other cellular component or mRNA obtained from the nucleus or cytosol.

Where the physiological parameter is for example the phosphorylation level of the marker, this may be determined by using labeled antibodies against phosphorylated substances such as labeled anti-tyrosine antibodies or antibodies which are capable of binding to phosphorylated GSK-beta..

Where the level of the tested parameter is localization in various cellular components, the amount of the marker in each compartment, or ratio of the amounts in various components may be determined. This may be done by separating the cellular components (for example lysing the cell and obtaining separately the membrane and the cytosol) or obtaining separately the cytosol and the nucleus and determining the protein content of the relevant biological marker in each separated cellular component s, by using any one of the methods mentioned above or other methods used to determine protein contents.

Alternatively it is possible for determining A3AR localization, to use labeled A3AR binding agents (antibodies, agonists, antagonists) especially fluorescent labeled binding agents, and monitor the localization of the A3AR on the surface of the cells, for example using a confocal microscope.

In general, the physiological parameter may change in one of two manners as compared to control: a change indicative of increased proliferation (herein after "pro-proliferative") as a result of treatment such as administration of the drug (preferably an A3AR modulator, most preferably an A3AR agonist), or a change

indicative of decreased proliferation (hereinafter: "anti-proliferative") as a result of treatment such administration of the drug.

Changes indicative of anti –proliferative effect of the treatment

(1) In the expression level: decrease in protein or mRNA expression of A3AR, PKB/Akt, PKA, β-catenin, c-myc, cyclin D1 and NF-$_\kappa$B, or increase in the protein or mRNA level of GSK-3β

(2) In phosphorylation level: I decrease in phosphorylation level of GSK-3β , increase in the phosphorylation level of PKB/Akt and PKA, and of β-catenin.

(3) In localization: decrease in the localization of A3AR receptor in the cellular membrane as compared to control, decrease in the localization of β-catenin in the nucleus as compared to cytosol. Decrease in the localization of NF-kappaB, in the nucleus as compared to the cytosol

Changes indicative of pro-proliferative effects of the treatment include:

(1) In the expression level: increase in protein or mRNA expression of A3AR, PKB/Akt, PKA, β-catenin, c-myc, cyclin D1 and NF-$_\kappa$B, or decrease in the protein or mRNA expression level of GSK-3β

(2) In phosphorylation: increased in phosphorylation level of GSK-3β decrease in the phosphorylation level of PKB/Akt and PKA and of β-catenin.

(3) In localization,:increase in the localization of A3AR receptor in the cellular membrane as compared to control, increase in the amount of β-catenin and NF-kappaB in the nucleus as compared to cytosol.

The changes indicative of a decreased proliferation show effectiveness of a drug , preferably an A3AR modulator, most preferably an A3AR agonist, administered for the treatment of a disease state wherein a therapeutically beneficial effect may be evident by decrease or inhibition of proliferation. Examples of such diseases that are typically characterized by excess proliferation

include, without being limited to, all types of cancer; and in particular all types of solid tumors; skin proliferative diseases (e.g. psoriasis) .

The term *"solid tumors"* refers to carcinomas, sarcomas, adenomas, and cancers of neuronal origin and if fact to any type of cancer which does not originate from the hematopoeitic cells and in particular concerns: carcinoma, sarcoma, adenoma, hepatocellular carcinoma, hepatocellularcarcinoma, hepatoblastoma, rhabdomyosarcoma, esophageal carcinoma, thyroid carcinoma, ganglioblastoma, fibrosarcoma, myxosarcoma, liposarcoma, cohndrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphagiosarcoma, synovioama, Ewing's tumor, leimyosarcoma, rhabdotheliosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, renal cell carcinoma, hematoma, bile duct carcinoma, melanoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small lung carcinoma, bladder carcinoma, epithelial carcinoma,glioma, astrocyoma, medulloblastoma, craniopharyngioma, ependynoma, pinealoma, retinoblastoma, multiple myeloma, rectal carcinoma, thyroid cancer, head and neck cancer, brain cancer, cancer of the peripherial nervous system, cancer of the central nervous system, neuroblastoma, cancer of the edometrium, as well as metastasis of all the above. It has been shown in accordance with the invention that increased expression of A3AR can be found not only in the primary tumor site but also in metastasis thereof.

Where the disease is cancer the cells that are obtained from the subject may be cells suspected of being transformed as well as other cells notabely blood cells such as neutrophiles. Cells suspected of being transformed may be obtained by methods known for obtaining *"suspicious"* cells such as by biopsy, needle biopsy, and the suspicion of being in a disease state, may be raised due to various imaging (NMR, MR, scanning, ultrasound, memographic) or pathological techniques. Cells of the blood may be obtained simply by drawing blood.

The changes indicative of an increased proliferation show effectiveness of a drug (preferably an A3Ar modulator, most preferably an A3Ar agonist) administered for the treatment of a disease or a condition wherein a therapeutically beneficial effect may be evident by increase proliferation. These conditions are typically conditions were normal cells die as a results of trauma (injury, ischemia, hypoxia) or as a result of administration of a toxic substances, or as a result of toxic treatment such as substances or radiation administered during the course of chemo-or radio-therapy. In particular this term refers to the effect of myelo-protective therapy- i.e. prevention in the decrease of the number of neutrophiles , and other white blood cells following chemo-or radio-therapy.

The drug is typically a drug that works via modulation of the A3AR associated signal transduction pathway and in particular is an A3AR modulator. An A3AR modulator in the context of the present invention refers to an A3AR agonist or antagonist and in accordance with a preferred example is an A3AR agonist.

The term *"ARAR-associated signal transudation pathway"* concerns any pathways which begins by activation by the A3AR receptor and continues by the effect of its down stream effectors . Currently it is known that these effectors include elements of the Wnt pathway and the NF-κB pathway, but further elements are constantly discover and this term may cover the newly found elements as well as well as newly found pathways associated with A3AR activation.

The sample refers to cells, tissue samples or cell components (such as cellular membranes or cellular components) obtained from the treated subject. By one embodiment the sample are cells known to manifest the disease ,for example, where the disease is cancer of type X, the cells are the cells of the tissue of the cancer (breast, colon, skin, liver, lungs, cells, etc.) or metastasis of the above . By another embodiment the sample may be non-disease cells such as cells obtained from blood for example neutrophiles .

The *"diseased state"* wherein effectiveness is indicated by decrease proliferation, includes tumor, and in particular solid tumors, example of solid

tumors being melanoma, colon carcinoma, prostate carcinoma, lung cancer, breast cancer, pancreatic cancer, skin proliferative diseases, such as psoriasis.

The disease state wherein the effective is indicated by increased proliferation, are in particular diseases wherein there is an increase in the blood counts of white cells such as neutrophiles as a result of chemo-or-radio therapy..

Monitoring the levels of at least one physiological parameter of the biological marker in the cells, in accordance with one of the uses of the above method, may also help to screen for likely candidates for treatment of a diseases state, notably for the treatment of cancer, most notably for the treatment of cancer through modulation of A3AR activity or through the modulation of A3AR signal transduction pathway, and in particular by the administration of A3AR agonist.

For example, it is possible to use cell cultures of the diseased state, for example, a specific line of cancer cells for which it is desired to administer the cells with drug candidates, such as anti-cancer candidates, and to determine whether the A3AR level has changed in cells treated with a specific drug-candidate to indicated an anti-proliferative effect.

In accordance with the invention it has been found that there is temporal fluctuations in the level of expression and in the cellular localization of the various biological markers after the administration of the A3AR modulator, and in particular A3AR agonist. This means that if ,for example, the amount of the protein of a specific marker decreased as a result of A3AR-modulator administration, this decrease may be most prominent X minutes after administration of the A3AR modulator, while after 2X minutes the decrease may be less prominent and after 3X minutes the decrease may be prominent again. This fluctuation behavior (typically in a sinusoidal-like pattern) characterizes the protein level of most biological markers as well as the localization on the cellular membrane of the A3AR receptors itself. It is clear that it is best to carry out the method of the invention after a time period from administration of the drug wherein the change in the level of the parameter is most prominent as compared to control.

Thus, before carrying out the method of the invention it is important to carry out a preliminary determination to establish the optimal time after the drug administration wherein the changes in the physiological parameters are most prominent as compared to control. This is achieved by administering the drug and monitoring the temporal changes in the physiological parameters (in vitro, in vivo, but most preferably in a series of subject wherein the sample is obtained at varying time periods after administration, and choosing as the time for determination the period wherein the difference between the parameters in the treated (cells, tissue, etc) are the largest as compared to the untreated control.

BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Figs. 1a-1c shows confocal laser microscopy analysis of B16-F10 melanoma cells labeled with the primary and secondary antibodies against A3AR and the Cy3-conjugated anti-goat IgG, respectively; wherein Fig. 1a exhibit an image of A3AR in untreated melanoma cells; Fig. 1b exhibit IB-MECA treated cells; and Fig. 1c exhibit a combined treatment with IB-MECA and MRS an A3AR antagonist. Images represent the center section of the X-Y plane.

Fig. 2 shows confocal laser microscopy analysis of B16-F10 melanoma cells labeled with the primary antibody against A3AR and the secondary antibody, Cy3-conjugated anti-goat IgG, respectively, exposed for different time periods to IB-MECA. Images represent the center section of the X-Y plane.

Fig. 3 shows Western blot analysis of A3AR internalization in untreated melanoma cells (control), or upon trypsin treatment, IB-MECA treatment, or a combined treatment with IB-MECA and trypsin. The level of the housekeeping protein β-actin did not change.

Figs. 4a-4b show Western blot analysis of A3AR in B16-F10 melanoma cells; wherein Fig. 4a presents results upon treatment of the cells for different time

periods with IB-MECA; and Fig. 4b presents results upon treatment with IB-MECA, a combination of IB-MECA and cycloheximide (protein synthesis inhibitor) or a combination of IB-MECA with MG132 (protein degradation inhibitor), as compared to untreated cells (control

Figs. 5a-5b show the correlation between tumor size and the level of regulatory elements in colon carcinoma cells, wherein Fig. 5a presents tumor size after 15 days of daily treatment with IB-MECA of mice inoculated with B16 melanoma cells while Fig. 5b presents the modulation of cell growth regulatory proteins (PKAc, PKB/akt, GSK-3β,β-catenin cyclin D1 ,c-myc and NF-κB) in the tumor lesions described in Fig. 5a (left prior to treatment with A3Ar agonist; right after treatment with A3Ar agonist).

Fig 6. shows immunoblot analysis of proteins extracted from prostate carcinoma cells, being A3AR, NF-κB, c-myc and cyclin D1 is in the presence (right lane) and absence (left lane) of IB-MECA.

Fig. 7. shows immunoblot analysis of protein extracts derived from colon carcinoma cell, being PKAc, PKB/Akt, β-catenin, c-myc and cyclin D1 NF-κB and GSK-3β in the presence (right lane) and absence (left lane) of IB-MECA.

Figs. 8a-8c show Western blot analysis of receptor functionality in B16-F10 melanoma cells determined by monitoring the level of PKA and GSK-3β; wherein Fig. 1a presents the effect of IB-MECA on PKA and GSK-3β levels in the melanoma cells, at different time periods; Fig. 1b presents the effect on cells treated simultaneously with IB-MECA+MRS 1523 and Fig. 1c presents the effect on cells treated with Forskolin or 8-Br-cAMP.

Figs. 9a-9b show the correlation between tumor size and the level of regulatory elements in colon carcinoma cells, wherein Fig. 9a presents tumor size after 15 days of daily treatment with IB-MECA of mice inoculated with HCT-116 human colon carcinoma as a function of time; while Fig. 9b presents the

modulation of cell growth regulatory proteins (A3AR, GSK-3β, cyclin D1 and c-myc) in the tumor lesions described in Fig. 9a.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the finding that there exists a cross-talk between A3AR and the Wnt and NF-κB signaling pathways. A3AR activation was found in cancer cells, to inhibit PKA, and PKB/akt thereby retaining GSK-3β in its active non-phosphorylated form [4]. Active (non-phosphorylated) GSK-3β was shown to phosphorylate and inactivate β-catenin, eliminating its migration to the nucleus and consequently inducing the down-regulation of c-myc and cyclin D1 [5], resulting in decreased proliferation of the cells.

In the NF-κB pathway the decrease in PKB/AKT level leads to down-regulation of IKK and NF-κB which will prevent the release of the latter from its complex with IKB and its entry to the nucleus, thus preventing the translocation of NF-κB to the nucleus, resulting in a decreased induction the transcription of cyclin D1 and c-myc, leading to a decreased in proliferation.

In proliferative diseases such as in tumor , including melanoma, colon carcinoma, prostate carcinoma as well as in other tumor cells, the untreated course of events , in manifested by changes in the physiological parameters of the biological markers in the pro-proliferative direction including failure of the phosphorylated GSK-3β to phosphorylate β-catenin, which thus accumulates in the cytosol. It then translocates to the nucleus where it induces the transcription of cyclin D1 and c-myc, leading to cell cycle progression [6-7]. Successful anti-proliferative treatment, monitored by the determination of the following changes in the level of the physiological parameters of the biological markers is a change of the physiological parameters of the biological markers in the anti-proliferative direction

The following are changes in the physiological parameters that mark successful treatment of disease wherein a beneficial effect is evident by decreased proliferation such as cancer:

(1) In the expression level: decrease in protein or mRNA expression of A3AR, PKB/Akt, PKA, β-catenin, c-myc, cyclin D1 and NF-$_\kappa$B, or increase in the protein or mRNA level of GSK-3β

(2) In phosphorylation level: decrease in phosphorylation level of GSK-3β , increase the phosphorylation level of PKB/Akt and PKA and beta-catenin .

(3) In localization: decrease in the localization of A3AR receptor in the cellular membrane as compared to control, decrease in the localization of β-catenin and NF-kappa B,in the nucleus as compared to cytosol.

The following are changes in the physiological parameters of the biological markers that are indicators of successful treatment of diseases and conditions wherein a therapeutically beneficial effect may be evident by the increase of proliferation such as increase in the proliferation of white blood cells following chemo-or radio-therapy:

(1) In the expression level: increase in protein or mRNA expression of A3AR, PKB/Akt, PKA, β-catenin, c-myc, cyclin D1 and NF-$_\kappa$B, or decrease in the protein or mRNA expression level of GSK-3β

(2) In phosphorylation: increase in phosphorylation level of GSK-3β ,decrease in the phosphorylation level of PKB/Akt and PKA and beta –catenin..

(3) In localization, :increase in the localization of A3AR receptor in the cellular membrane as compared to control, increase in the amount of β-catenin and NF-kappaB in the nucleus as compared to cytosol.

SPECIFIC EXAMPLES

Materials and Methods

Rabbit polyclonal antibodies against murine and human A3AR, PKAc, c-myc and GSK-3β were purchased from Santa Cruz Biotechnology Inc., Ca, USA.

Rabbit polyclonal antibodies against murine and human cyclin D1 (Upstate, NY), A_{2B} adenosine receptor, Cy3-conjugated anti-goat IgG and Fluorescein-conjugated anti-rabbit IgG were purchased from Chemicon, Ca.

Cycloheximide and Forskolin was obtained from Sigma, St Louis, and 8-Br cAMP and MG132 from Calbiochem, Ca.

Male ICR mice aged 2 months, weighing an average of 25g as well as Nude/BalbC male, 10 weeks old mice (Harlan Laboratories, Jerusalem, Israel), were employed in the following experiments. Standardized pelleted diet and tap water were supplied.

Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Rabin Medical Center, Petah Tikva, Israel.

B. Immunostaining and confocal microscopy

B16-F10 melanoma cells were grown for 24h on cover slips coated with Ploy-L-Lysine (500μg/ml). Cells were fixed in 4% formaldehyde in phosphate–buffered saline (PBS) for 1 hour (h) at room temperature. The fixed cells were rinsed three times for one min with PBS. To block nonspecific interaction of the antibodies, cells were incubated for 30 min in 4% normal goat serum (NGS) in PBS (1% bovine serum albumin (BSA), 0.1% Triton X-100). For the single labeling experiments, cells were then incubated with the primary antibodies against A3AR at a dilution of 1:1000 in PBS (1% BSA, 1% NGS, 0.1% Titon X-100) for 24 h at 4°C. For the double labeling experiments, antibodies against the A_{2B} adenosine receptor ($A_{2B}R$) were added at a dilution of 1:1000 to the reaction mixture. After being washed three times for 3 min with PBS, cells were incubated with Cy3-conjugated anti-goat IgG for the single labeling experiment, and with both, Fluorescein-conjugated anti-rabbit IgG and Cy3 for the double labeling. Both antibodies were diluted to 1:250 in PBS and incubated in the dark for 2 h. Cells were rinsed with PBS three more times and mounted with AM 100 (Chemicon, Ca). Stained cells were visualized by Confocal Microscope (Zeiss, Axiovert 100 M,

excitation at 553 and emission at 560-615nm for Cy3, and 492 and 520 nm respectively, for Fluorescein).

B. Western Blot Analysis

To detect the level of expression of the A3AR proteins Western blot analysis was performed. Cells were incubated in the presence and absence of IB-MECA (10nM), MRS1523 (10nM), Forskolin (50nM), 8 Br cAMP (100μM), Cycloheximide 20μg/ml or MG 132 (20nM) for different time periods at 37°C. Cells were then rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50mM Tris buffer pH=7.5, 150mM NaCl, NP 40). In the experiment examining the effect of trypsin on A3AR expression, cells were incubated with 0.5 ml of 0.25% trypsin for 5 minutes. The trypsinized cells were washed again with ice-cold PBS, harvested by centrifugation and subjected to lysis in TNN buffer. Cell debris was removed by centrifugation for 10 min, at 7500xg. The supernatant was utilized for Western Blot analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50μg) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 1% bovine serum albumin and incubated with the desired primary antibody (dilution 1:1000) for 24h hour at 4°C. Blots were then washed and incubated with a secondary antibody for 1h at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison, W1, USA). Data presented in the different figures are representative of at least three different experiments.

D. Northern Blot Analysis

Total RNA was isolated from B16-F10 melanoma cells treated with IB-MECA, utilizing Tri–reagent (Sigma, Saint –Louis). The samples were then subjected twice to phenol:chloroform extraction and washed with chloroform. RNA was precipitated with sodium acetate/ethanol following washing with ethanol, then

denatured, separated (25 μg per lane) in 1.1% formaldehyde agarose gels and transferred to Hybond-N membrane. The 390 bp *EcoR*I fragment from A3AR cDNA clone of mouse (TAA3I.S), kindly supplied by Dr Kathia Ravid, was prepared by random-primed synthesis. Probes were used in RNA blot analysis at a hybridization temperature of 42°C in the presence of 50% formamide.

E. RT-PCR-for detection of intact RNA in formalin-fixed paraffin-embedded tissues.

Tissue sections (5μm thick) on slides that ,stained by H&E were observed by a pathologist. The neoplastic area and the normal area were detected and each one marked separately. The neoplastic tissue and the normal tissue were collected to different microcentrifuge tubes. The samples were treated with proteinase K at a final concentration of 0.1 mg/ ml and incubated at 370C for 1h to allow for DNA digestion. Cells lysate were heated to 95^{0}C for 15 min in order to inactivate DNase and proteinase K. Following centigugation at 14,000 RPM for 5 min, 17ul of the supernatant was transferred to separate tube and 4 μl of RT mixure [5mM dNTPs, 2.5 μM random hexamer, 5 U RNasin, 100 U SuperScript One Step RT-PCR with Platinum Taq (Invitrogene) were added.

The RT reaction was performed at 45^{0}C for 45 min. The PCR reaction was changed dependent on the primers used for the amplification, for set No I. the RT was followed by heating to 99^{0}C for 5 min , 50 cycles of 94^{0}C for 30s, 59^0 C for 45s and 73^{0}C for 45s were performed. For set No II, the annealing was done at 55^{0}C . Products were elecrtophoresed on 2% agarose gels , stained with ethidium bromide and visualized with UV illumination. The specificity of the RT-PCR reaction was confirmed by size determination on agarose gels in comparison to a positive control , from RNA extracted using standard techniques and by sequencing the RT-PCR product and comparing the sequences to the known sequences (ADORA3-L77729, L77730).

Table1. Nucleotide sequence of A3 primers

Gene	Fragment size	Primer sequence (set No I)
A3AR	153	5'-ACGGTGAGGTACCACAGCTTGTG
(Human ADORA3)		3'-ATACCGCGGGATGGCAGACC
	361	5'-ACCCCCATGTTTGGCTG (set No II)
		3'-GCACAAGCTGTGGTACCTCA

F. In vivo studies

Mice were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Rabin Medical Center, Petah Tikva, Israel.

Melanoma: C57BL/6J, male mice (Harlan Laboratories, Jerusalem, Israel) aged 2 months, weighing an average of 25g were used. B16-F10 (2.5x105) melanoma cells were subcutaneously (s.c.) injected to the flank of the mice. IB-MECA at a dose of 100μg/kg body weight was administered orally once daily, starting 24h after tumor cells' inoculation. The control group was treated daily, orally, with the vehicle only. Mice were sacrificed after 15 days, tumor lesions were excise and protein was extracted for measurements as described above. The tumor

size (width (W) and length (L)) was measured with a caliber and calculated according to the following formula: Tumor Size = (W)2xL/2. Each group contained 15 mice and the study was repeated 3 times.

Colon carcinoma: Nude/BalbC male, 10 weeks old mice were used. HCT-116 Human colon carcinoma cells ($1.2x10^6$ cells in 100μL PBS) were inoculated subcutaneously to the flank of the mice. Treatment was initiated when tumors reached a size of ~150mm^3. Each group contained 10 mice, the control group treated with vehicle only, while the test group was administered daily orally with IB-MECA (10 μg/kg).

After 32 days the mice were sacrificed, tumor lesions were excise and protein was extracted for measurements as described above. Tumor size was evaluated by measuring with a caliber width (W) and length (L) and calculated according to the above formula.

G. Statistical analysis

The results were evaluated using the Student's t-test, with statistical significance at $p<0.05$. Comparison between the mean value of different experiments was carried out.

Example 1: Monitoring Changes in Biological Marker localization in Melanoma Cells

To study receptor localization, confocal laser microscopy was utilized. Untreated cells (control) highly exhibited A3AR on the cell surface, as seen from the fluorescence intensity level (Fig. 1a). A marked decrease in the fluorescence level was noted after 5 min in the IB-MECA treated cells (Fig. 1b). Exposure of the melanoma cells to the A3AR antagonist MRS1523, in the presence of IB-MECA, resulted in cell surface fluorescence intensity similar to that of the control

(Figure 1c). These data suggest that rapid receptor internalization took place upon IB-MECA treatment.

To further explore the time course kinetic of A3AR internalization, B16-F10 melanoma cells were exposed for different time periods to IB-MECA and confocal microscopy analysis was carried out. Figure 2 depicts the gradual internalization rate which occurred within a few minutes, resulting in the disappearance of the fluorescence after 6 minutes. Prolonged exposure (15 min) of the melanoma cells to IB-MECA, resulted in receptor externalization to the cell surface. This was followed by internalization/externalization after longer incubation time periods (30 and 60 min). To confirm the observation that the fluorescence level is decreased as a result of internalization, optical sectioning of the cells was performed (data not shown). In untreated cells (controls) the receptor was exhibited on the cell surface, and upon exposure to IB-MECA (for 5 min), it was presented in the cytoplasm, supporting the notion that A3AR translocates from the membrane to the cytosol. After 15 and 60 min exposure, the receptor was seen both in the cytoplasm and on the cell surface. In the 60 min samples, more fluorescence was seen in the cytosol in comparison to the 15 min. To show the specificity of A3AR localization, it was compared to that of the A_{2B} adenosine receptor. Interestingly, in the untreated cells, while A3AR was mainly localized on the cell surface, the A_{2B}R was seen in the cytoplasm. Upon exposure of the cells to IB-MECA, the A_{2B}R was masked by A3AR in the cytoplasm.

Example 2: Monitoring changes in RNA and protein expression level of A3AR in IB-MECA administered melanoma cells

Next, receptor internalization by treating melanoma cells with trypsin which removes the cell surface receptor, was examined. Untreated melanoma cells expressed a high level of A3AR protein, which was down regulated after IB-MECA or trypsin treatment (Fig. 3. lanes 1, 2 and 3). This indicates that in the control untreated tumor cells most A3AR is exhibited on the cell surface and is

subjected to digestion by trypsin. In the IB-MECA+trypsin treated cells (5 min), a negligible difference was observed between the trypsin treated and untreated cells, demonstrating that most A3AR was already internalized (change in localization) due to the treatment with the A3AR agonist IM-MECA, thus protected from digestion (Figure 3a., lanes 3 and 4).

Time dependent expression of A3AR in the melanoma cells was examined by Western blot analysis. IB-MECA induced modulation of A3AR expression in a sinusoidal pattern, i.e. downregulation and upregulation, occurred at different time points (Figure 4a.). This pattern indicted the optimal time for carrying out the method of the invention has to be determined experimentally.

To test whether protein expression was modulated due to degradation and re-synthesis, the cells were exposed for 180 min to IB-MECA in the presence of MG132 (protein degradation inhibitor) and cycloheximide (protein synthesis inhibitor). Indeed, MG132 prevented A3AR downregulation and cycloheximide inhibited receptor upregulation, illustrating that following internalization, receptor degradation and re-synthesis took place (Figure 4b). Moreover an increase in mRNA expression level was observed, suggesting that a de novo synthesis of A3AR had occurred (Figure 4c). The specificity of this response was demonstrated by MRS1523 which reversed the increase in mRNA expression

Example 3: Monitoring expression level of biological markers in melanoma inoculated mice treated with IB-MECA

IB-MECA markedly suppressed the development of B16-F10 melanoma tumor growth (76% inhibition, $p<0.0001$, Fig 5a). In tumor lesions excised from these mice, Western blot analysis (Fig. 5b) revealed that the levels of both PKA and PKB/Akt are decreased. Consequently, an increase in the total GSK-3β level was noted, thereby leading to a decrease in the level of β-catenin. Cyclin D1 and c-myc, known to be transcripted following translocation of β-catenin/Lef1 to the cell nucleus, were both found to be downregulated in the IB-MECA treated melanoma cells.

In addition, the level of the transcription factor NF-kB, which its activation plays an important role in tumor development was also down-regulated. The level of the housekeeping protein β-actin did not change (not shown).

Example 4: Monitoring expression level of biological markers during inhibition of colon carcinoma development in mice by IB-MECA treatment

IB-MECA markedly suppressed the development of colon carcinoma cells in mice inoculated with HCT-116 human colon carcinoma cells (Fig. 5a). In tumor lesions excised from mice inoculated with the HCT-116 human colon carcinoma cell, Western blot analysis revealed downregulation of A3AR, upregulation of GSK-3β expression level, followed by a decrease in the level of c-myc and cyclinD1 (Fig. 5b). The level of the housekeeping protein β-actin did not change

Example 5: Monitoring change of expression of Biological markers in Prostate Carcinoma (PC-3)

The effect of IB-MECA on key proteins downstream to A3AR activation in prostate carcinoma cells was also examined in a manner similar to that performed with melanoma cells (see above). Fig. 6 presents immunoblot analysis of proteins extracted from prostate carcinoma cells, wherein downregulation of A3AR, NF-κB, c-myc and Cyclin D1 is exhibited.

Example 6: Monitoring changes of expression level of biological markers in Colon Carcinoma cells as a result of IM-MECA administration

The effect of IB-MECA on key proteins downstream to A3AR activation in HCT-116 human colon carcinoma cells was also examined in a manner similar to that performed with melanoma cells (see above). Fig. 7 presents Immunoblot analysis of protein extracts derived from colon carcinoma cell, wherein treatment with IB-MECA (right lane) caused downregulation of PKAc, PKB/Akt, β-catenin, c-myc and cyclin D1 and NF-κB and upregulation of GSK-3β expression level as

comared to control (left lane). These results conform with the results obtained with melanoma, and prostate cancer cells and presented hereinabove and support the notion that determination of the expression level of these regulatory elements may function as biological markers for disease states.

It should be noted that the level of the transcription factor NF-kB, which its activation plays an important role in tumor development was also downregulated.

Example 7: Monitoring expression of key proteins downstream to A3AR activation by IM-MECA treatment in melanoma cells

To test receptor functionality, the protein expression level of PKA and GSK-3β, which are modulated downstream to A3AR activation, was examined. Functional receptor sensitization by IB-MECA was observed after 15 and 60 min and was manifested by decreased PKA and increased GSK-3β levels. However, at 30 min, PKA level stabilized and GSK-3β only slightly increased, indicating that receptor de-sensitization/re-sensitization took place upon chronic exposure to the agonist . The specificity of this response was demonstrated by introducing MRS1523, forskolin and 8-Br-cAMP, all known to counteract A3AR activation. Indeed, the modulatory effect of IB-MECA on PKA and GSK-3β was reversed in the presence of the above agents (Fig 8a, 8b and 8c).

Example 8: IB-MECA inhibits colon carcinoma development in mice and down regulates expression of biological markers

IB-MECA markedly suppressed the development of colon carcinoma cells in mice inoculated with HCT-116 human colon carcinoma cells (Fig. 9a). In tumor lesions excised from mice inoculated with the HCT-116 human colon carcinoma cell, Western blot analysis revealed downregulation of A3AR, upregulation of GSK-3β expression level, followed by a decrease in the level of c-myc and cyclinD1 (Fig. 9b). The level of the housekeeping protein β-actin did not change (data not shown).

Example 9: Monitoring the expression level of biological markers in clinical treatment

The purpose of the following study is to determine the ability of IB-MECA at varying doses to alter the expression profile of relevant biological markers in subjects with newly diagnosed colorectal cancer. The subjects will be tested for the level of mRNA of the following markers prior to treatment by performing RT-PCR on tissue obtained in the diagnostic biopsy.

At least one, but preferably several of the following tumor markers are determined: , A3AR receptor ,PKA ,PKB/Akt ,GSK-3β ,β-catenin. Cyclin D1 ,c-myc, NF-kB, .

Subjects diagnosed include patients with colorectal lesions felt to have high likelihood of being malignant and who will most likely undergo biopsy followed by definitive surgery.

Biopsy specimens are removed from subjects (through colonoscopy) and undergo northern separation or RT-PCR amplification prior to treatment for testing for the above biological markers..

Treatment regimen: Cohorts of 5 to 10 patients are treated at escalating doses of IB-MECA, either daily or twice a day. Treatment with IB-MECA is initiated before definitive surgery.

After a set time of treatment with IB-MECA (a time which was previously determined to show maximal difference between the treatment and control) tumor lesion is removed at surgery and A3 receptor mRNA expression level along with downstream signals , ,PKA ,PKB/Akt ,GSK-3β ,β-catenin. Cyclin D1 ,c-myc, NF-kB, is determined by using RT-PCR . The level of expression of the different proteins will be compared to that determined from the biopsy specimen prior to treatment.

In the same manner, the effect of treatment with IB-MECA, via determination of the level of regulatory markers, is determined in cases of breast cancer, prostate cancer, melanoma and others.

Example 10: Detection of A3AR receptor on human Neutrophils

10x106 Neutophils cells isolated from 20 ml of human blood were incubated for 15 min with 0.01 mM or 10 mM of CF101 at 370C. The cells were collected by centrifugation and washed with PBS . RNA was extracted from the cells by using TRI-reagent (Sigma). RNA level was quantified using spectrophotometer and 1mg from each sample were subjected to RT-PCR using SuperScript One Step RT-PCR with Platinum Taq (Invitrogene) , as described above in section E., by using the set No II as primers for amplification of 361 bp fragment. RT-PCR products were detected by elecrophoresis and the size was verified by comparing with known RNA.

The results are shown in Fig. 10. As can be seen the A3AR agonist IB-MECA was able to increase the expression of ARAR in neutrophiles. This indicates that the neutrophiles responded, in a pro-proliferative manner, to the therapeutical treatment. Therefor detecting changes in the level of A3AR can indicate effectiveness of a treatment (by an A3AR agonist) for increasing neutrophile count for example to counter react the effect of chemotherapy.